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STUDIES ON SYNTHESIS AND UTILIZATION OF FORMOSE
PART II. CHROMATOGRAPHIC FRACTIONATION AND IDENTIFICATION OF FORMOSE SUGAR

Takashi Mizuno, Masakatsu Asai,
Akira Misaki and Yoshinobu Fujihara

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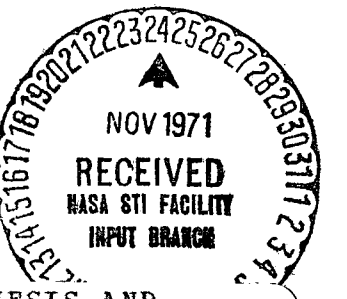

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STUDIES ON SYNTHESIS AND UTILIZATION OF FORMOSE
PART II. CHROMATOGRAPHIC FRACTIONATION AND IDENTIFICATION OF FORMOSE SUGAR

T. Mizuno, M. Asai, A. Misaki[1] and Y. Fujihara[2]

ABSTRACT: The sugar composition of formose, that is, monosaccharide mixture produced from the dilute formaldehyde aqueous solution at yellowing point in the presence of calcium hydroxide, was investigated by the various methods of chromatography, i.e., paper chromatography of formose, thin-layer chromatography of formose and its phenyl osazone derivative, gas-liquid chromatography of alditol acetate and trimethylsilyl derivatives of formose, and liquid-solid chromatography (sugar-autoanalyzer) of formose sugar. The results were as follows:

Formose was mixture of monosaccharides of various carbon number (aldose and ketose). Hexose was the major component, pentose was the second and triose, tetrose and heptose were the minor components. The following 12 kinds of DL-racemic sugars in the above-mentioned monosaccharide group were detected. Glycerose and dioxyacetone as triose; erythrose and threose as tetrose; arabinose and xylose as pentose; mannose, galactose, glucose, gulose, fructose and sorbose as hexose group, respectively. A number of other pending spots or peaks were found on chromatographic patterns of formose.

In our previous report [1], we confirmed the formation of sugars (formose) /344* by the aldol condensation of formaldehyde in an aqueous solution in the presence of an inorganic or organic base. We reported that, $Ca(OH)_2$ was the best condensation agent of all the bases, that there is a sugar forming peak (yellowing point), that formose is a mixture of various monosaccharides such as the hexoses and pentoses, etc., and that the sugar composition is similar without regard to the type of base used. For the purpose of the present report, an attempt was

[1]Faculty of Agriculture, Shizuoka University, Institute of Scientific and Industrial Research, Osaka University.

[2]Faculty of Agriculture, Shizuoka University, Hitachi Co., Ltd., Naka Manufactory

*Numbers in the margin indicate pagination in the foreign text.

made to fractionate and confirm the sugars by paper (PPC), thin film (TLC), gas (GLC) and liquid (LSC) chromatography. Comparisons of these methods are made with the intent of studying the sugar compositions of formose in more detail.

EXPERIMENTAL METHODS AND RESULTS

1. Standard Sugar

C_3 sugars: D-glycerose was prepared by the lead tetraacetate oxidation of D-fructose [2]. $[\alpha]_D^{15}$ + 8.5° (c = 0.5, water).

C_4 sugars: D-erythrose was prepared by the lead tetraacetate oxidation of D-glucose [2]. $[\alpha]_D^{15}$ - 31.0° (c = 0.5, water).

C_5 sugars: Commercial, special grade D-ribose, L-arabinose and D-xylose were used.

C_6 sugars: L-rhamnose, D-glucose, D-mannose, D-galactose, D-fructose and L-sorbose were all commercial products of the special reagent, or first class grade.

C_7 sugars: D-glycero-D-gulo-heptose and D-glycero-D-indo-heptose were synthesized from D-glucose by the cyanhydrin method [2]. The sedohepturose [Translator's note: phonetic in the original] and the D-manno-hepturose were donated by Dr. N. K. Richtmyer [3] and the cholitose (D-altro-3-hepturose) and the D-gluco-hepturose were donated by Dr. Hirao Okuda [4]. /345

2. Synthesis of Formose

Based on the method covered in the previous report, [1], 450 ml of commercial formalin (37%, S. G. 1.1141, 500 g, special grade) were taken together with two liters of water and 500 ml of methanol in an Erlenmeyer flask. This was held in a 50°C bath while being agitated and 30 g of calcium hydroxide added. It was then violently agitated, causing it to reach the yellowing point (about 20 minutes). The mixture was then immediately neutralized with 160 ml of 20% sulfuric acid. After being made slightly acidic, it was filtered to remove the precipitate.

After neutralizing the filtrate with an excess of calcium carbonate powder, 4-5 g of activated charcoal were added. This was heated at 80° for 30 minutes, then filtered. The filtrate was reduced-pressure concentrated to 1/3 of its volume, then processed four times through an Amberlite IR-120 B (H^+) column (2 liter) then an Amberlite IRA-410 (OH^-) column (2 liters), in that order, to produce the refined sugar solution.

This sugar solution was reduced-pressure concentrated below 60° into a 80% syrup which was used for the experiments. The syrup was clear and colorless. It had a strong sweet taste, like that of rice syrup, and a water content of 20.4%. $[\alpha]_D^{15}$ 0° (c = 5, water).

The yield was 1,411 g of 80% syrup from 5 kg of 37% formalin. The yield per unit of CH_2O converted to glucose was about 53%.

3. Fractionation by PPC

The results shown in Figure 1 were obtained.

4. Fractionation by TLC

The results shown in Figure 2 were obtained.

5. Fractionation of Phenyl Osazones by TLC /346

(1) Preparation of the phenyl osazones of the standard sugars: Into a 15-ml capacity centrifuge tube were placed 1 millimole of sugar, 3 millimoles of phenylhydrazine hydrochloride, 3.5 millimoles of sodium acetate and 8 ml of water. A cooling glass tube was attached and the reaction carried out in a boiling water bath for 30 minutes. After cooling it was centrifuged (4,000 rpm, five minutes) and the mother liquid discarded. The precipitant was dissolved in a small quantity of hot methanol, then recrystallized by adding water slowly. The crystals were dried in a vacuum desiccator.

(2) The formose phenyl osazones: The formose phenyl osazone (A) was prepared and dried by the same method as that used for the standard sugars. (A) was treated with ether to divide it into a soluble portion (B) and an insoluble portion. The insoluble portion was dissolved in 10 times the volume of 80% hot methanol, then cooled and divided into the precipitate (D) and the soluble portion (C).

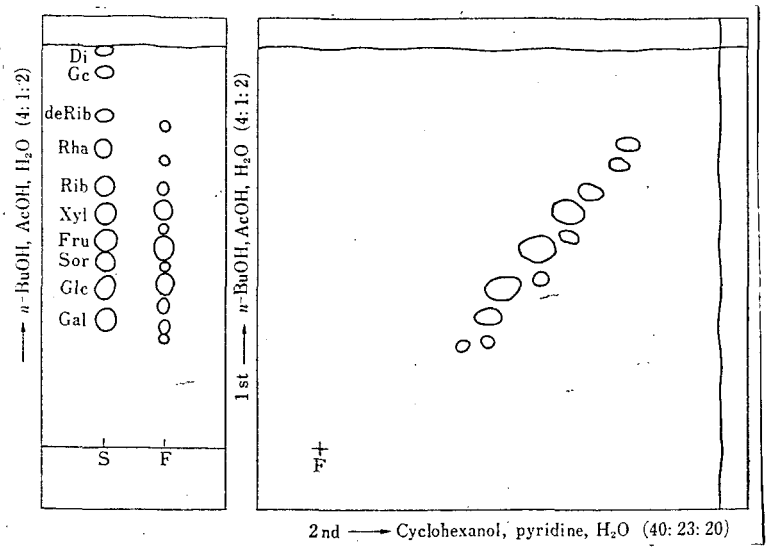

Figure 1. Paper Chromatograms of Formose Sugar. Filter paper: Toyo No. 50, 40x40 cm. Development method: multiple one or two dimensional ascending method (30°C, 2 times). Reagents: *p*-anisidine-HCL-ethanol and phloroglucinal-HCL-ethanol. S: authentic natural sugars, F: formose sugars. Di: dioxyacetone, Xyl: D-xylose, Gc: D-glycerose, Ara: L-arabinose, deRib: 2-deoxy D-ribose, Glc: D-glucose, Rib: D-ribose, Gal: D-galactose, Rha: L-rhamnose; Man: D-mannose, Ery: D-erythrose, Sor: L-sorbose, Hep: D-heptose, Fru: D-fructose.

(3) Differentiation by TLC: The comparison between the formose osazones and the standard sugar osazones gave the results shown in Figure 3. Two-dimensional development gave the TLC map for formose osazone shown in Figure 4.

(4) TLC and Densitometry: The relative densities of the formose osazones fractionated by TLC were measured with an Atago Optical Densitomaster Kemic Model S giving the results shown in Figure 5.

(5) Fractionation by TLC shavings: The components of each spot fractionated by TLC were extracted with hot methanol after scraping off 10 samples of each thin film spot. The filtrate was concentrated, /347
dried and powdered. The spot components obtained were again fractionated by TLC to give the results shown in Figure 6.

6. Fractionation by GLC

(a) As alditol acetates: Following the method of H. Björandal [5], 20 mg of formose (80% syrup) was dissolved in 2 ml of water then reduced with 40 mg of sodium borohydride for five hours at room temperature. Concurrently with /348
separating the excess sodium borohydride with Amberlite IR-120 (H^+) the cations were removed. After concentrating the solution, the methanol was removed together with the boric acid in the form of trimethylborate by repeated reduced-pressure distillation. The reduced substance obtained was acetylated by heating

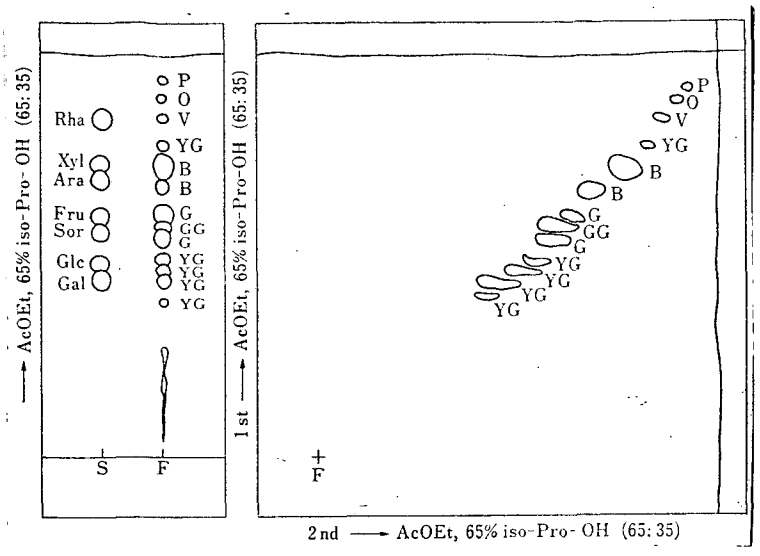

Figure 2. Thin-Layer Chromatograms of Formose Sugar. Thin-layer: Merck kiesel guhr G (0.05 N CH_3COON_a), 0.25 mm, 20x20 cm. Reagent: anisaldehyde-H_2SO_4. P: purple, G: green, O: orange, YG: yellowish green, V: violet, CG: greenish grey, B: blue.

for two hours at 100° with 0.5 ml of pyridine and 10.5 ml of acetic anhydride. It was then offered for GLC in that form, or after preparation in accordance with the conditions of J. S. Sawrdeker et al. [6] after dissolution in chloroform subsequent to concentration. The results are shown in Figure 7 and Table 1. Additionally, the standard alditol acetate was prepared by the same method from standard sugar.

(b) As TMS sugar: Based on the method of C. C. Sweely [7], 100 mg of formose (the anhydride) were dissolved in 10 ml of anhydrous pyridine, 2 ml of HMDS and 1 ml of TMCS added and mixed in. This was heated for 1-2 minutes at 60°-70°C, then allowed to stand for 10 minutes. After this, 30 ml of chloroform and 30 ml of water were added and mixed in by agitation. After standing or separating by centrifuge, the supernatant was discarded, the chloroform layer washed three times with water and the chloroform then removed under reduced pressure. After vacuum drying, the product was dissolved in 2 ml of chloroform or pyridine and 1-2 μl offered for GLC. The results were shown in Figure 8. Additionally, a check was made by selecting an internal standard from a Tokyo Kasei Company TMS-Sugar Kit and noting the increase in the size of the peak accompanying its addition.

7. Fractionation by LSC

Twenty mg of formose (80% syrup) were dissolved in 0.5 ml of water and subjected to analysis in a Hitachi Model 034 liquid-solid chromatograph (automatic sugar analyzer). Classification was attempted by comparison with a

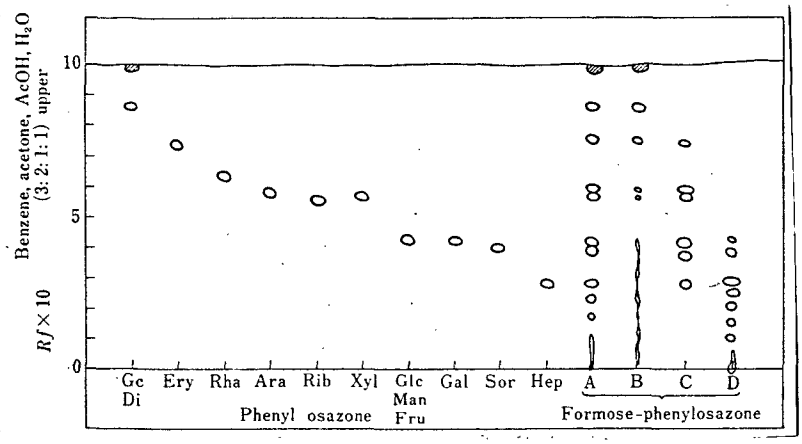

Figure 3. Thin-layer Chromatograms of the Phenyl Osazones Prepared from Natural Sugars and Formose Sugar. Fractionation of the formose phenyl osazone:

Formose phenyl osazone (A)

↓Et_2O

Et_2O-extract (B) | Residue

Residue → ↓10 vol. 80% MeOH, 80°C → ↓cooling, 5°C

Filtrate (C) | Ppt. (D)

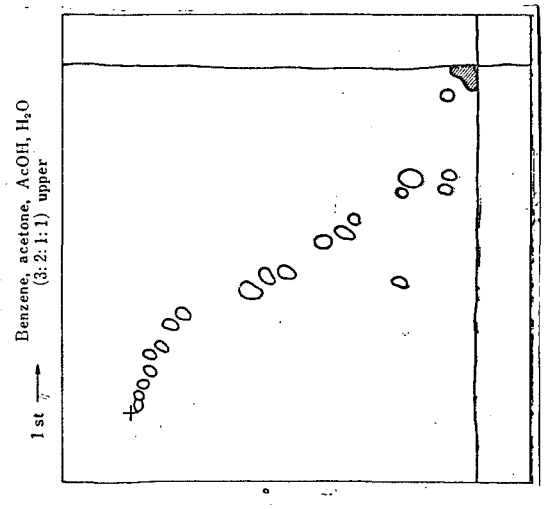

Figure 4. Thin-Layer Chromatogram of the Formose Phenyl Osazone. Thin-layer: Merck kiesel gel G, 0.25 mm, 20x20 cm.

chromatogram obtained for standard sugars under the same conditions. The results are consolidated in Figure 9.

DISCUSSION

It has been deduced principally from PPC that the formose produced by the aldol concentration of formaldehyde is a mixture of aldoses and ketoses of the C_2-C_8 sugars [1]. A quantitative study of the formose component sugars together with their classification would be desirable from the viewpoint of studying the formose formation mechanics and from the viewpoint of utilizing formose. /349

The sugar components of formose produced at the yellowing point when the quantity of sugar formed reaches a peak in the formaldehyde condensation reaction were subjected as free sugars or in the form of such derivatives as their osazones, aditol acetates or TMS sugars to various types of chromatographic analysis in an attempt to fractionate formose and to classify these sugars.

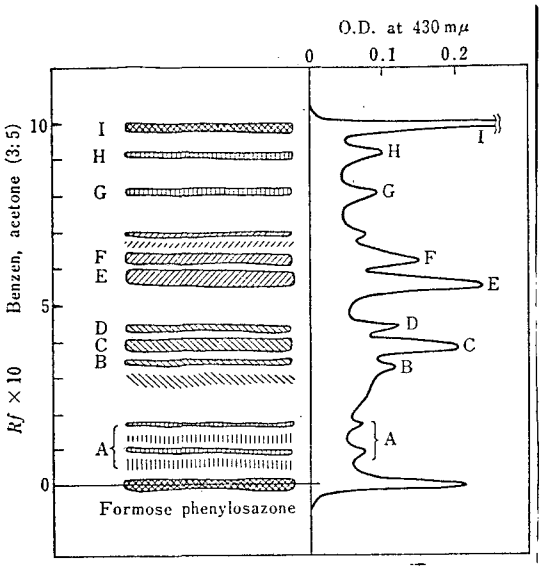

Figure 5. Thin-Layer Chromatogram and Its Densitogram of the Formose Phenyl Osazone. Thin-layer: Merck kiesel gel G. Development method: one dimensional multiple-ascending method (25°C, 2 times). Densitometer: Atago densitomaster kemic S, filter 430 mμ, slite 0.5x10 mm, range 0.5, chart speed 100 mm/min.

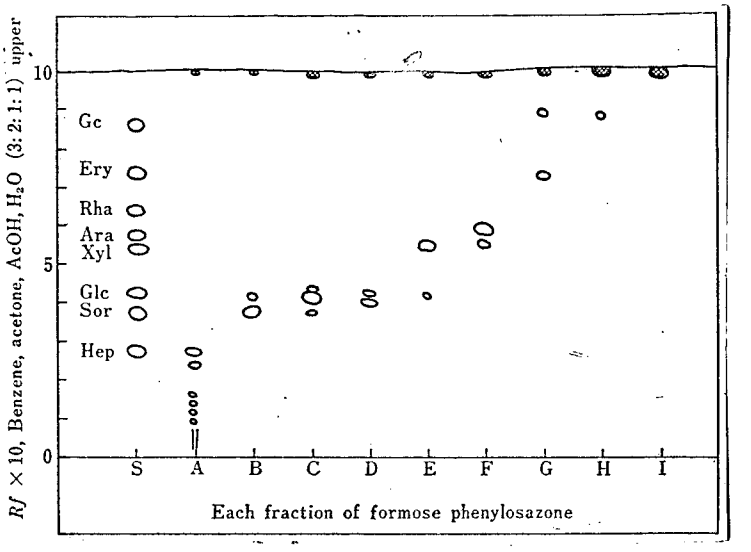

Figure 6. Thin-Layer Chromatograms of Each Fraction Obtained from the Formose Phenyl Osazone. Thin-layer: Merck kiesel gel G, 0.25 mm.

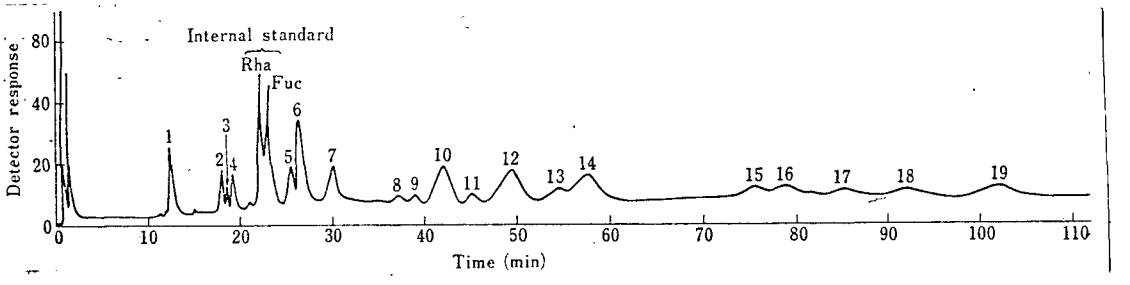

Figure 7. Gas-Liquid Chromatogram of Alditol Acetates Derived from Formose Sugar. Apparatus: Hitachi K-53 Gaschromatograph, detector FID. Column: 200xϕ0.3 cm stainless steel, 3% ECNSS-M on Gas Chrom Q. Temperature program: 80°→195°C (7.5°C/min). Carrier gas: N_2 40 ml/min.

Table I. RELATIVE RETENTION TIMES (T) OF AUTHENTIC ALDITOL ACETATES, AND FORMOSE AFTER REDUCTION AND ACETYLATION

Authentic alditol acetates	T	Molar response	Component from formose	T	Correspond to
Glycerol	0.56	0.88	1	0.56	Glycerol
Erythritol	0.81	**1.00**	2	0.82	Erythritol
Threitol	0.85	—	3	0.85	Tetritol
Rhamnitol	**1.00**	—	4	0.87	Threitol
Fucositol	1.05	—	5	1.14	Ribitol
Ribitol	1.17	—	6	1.18	Arabinitol
Arabinitol	1.18	1.26	7	1.35	Xylitol
Xylitol	1.35	—	8	1.65	Hexitol
Mannitol	1.74	1.29	9	1.74	Mannitol
Galactitol	2.05	1.26	10	1.84	Hexitol
Glucitol	2.24	1.26	11	2.05	Galactitol
			12	2.24	Glucitol
			13	2.47	Hexitol
			14	2.59	Hexitol
			15	3.40	Heptitol
			16	3.58	Heptitol
			17	3.84	Heptitol
			18	4.20	Heptitol
			19	4.66	Octitol ?

At the present state of sugar analysis technology, it is a difficult task to accurately fractionate, classify and quantitatively determine formose sugar which is said to be a mixture of more than 30 types of aldoses and ketoses having different carbon numbers. Initially, fractionation by PPC and TLC was attempted and generally more than 10 spots were produced; however, mutual separation was inadequate. Next, group isolation of the sugars by TLC after forming them into their phenyl osazone derivative was attempted. Relatively good separation between the sugars having different carbon numbers was observed. The sugars having the same C numbers with the same C-2 position epimers and 2-ketones give the same osazone; therefore, they could not be confirmed. When the monosaccharides in formose were reduced with sodium borohydrate into their sugar alcohols and GLC was performed on their acetates, sugar peaks corresponding to 19 types were detected (Figure 7). This method has the defect, however, that it is not possible to get rid of the 2-ketoses and differentiate the corresponding aldoses (the same is true about the other ketoses). An attempt was made to offset this by converting formose to the TMS derivatives and fractionating with GLC. The hexose and pentose peaks overlapped, however. When detection accurately increased, a number of peaks for each type of sugar such as α-, β-anomer [Trans. note: phonetic in the original], pyranose and pentose appeared. This method is not thought to be suitable for the classification of the sugar components of formose which is a mixture of polysaccharides because it is too complicated [8]. Finally, it was possible to fractionate peaks for 21 types of sugar when formose was subjected to LSC (automatic sugar analyzer) in the free sugar state (Figure 9).

Good separation appeared within the experimental limits; however, the problems of analytical equipment and time cannot be escaped.

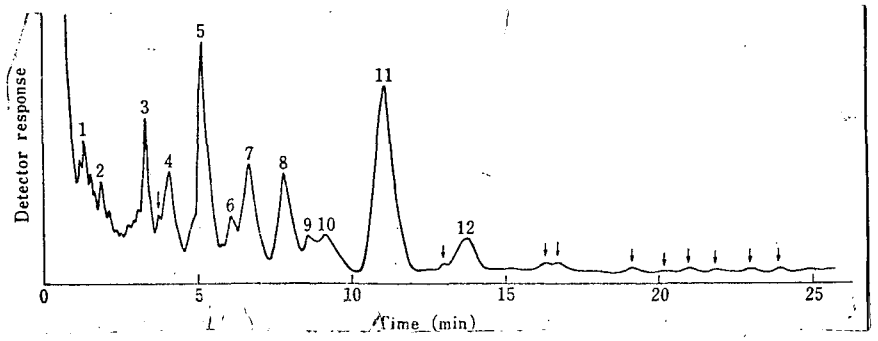

Figure 8. Gas-Liquid Chromatogram of Trimethylsilyl Derivative of Formose Sugar. Apparatus: Hitachi 063 Gaschromatograph, detector FID. Column: 200xϕ0.3 cm stainless steel, 3% SE-52 on Chromosorb W, 150°C. Carrier gas: N_2 70 ml/min. 2, erythrose; 4, arabinose, 5, xylose; 8, mannose; fructose; 10, gulose; 11, galactose, sorbose; 12, glucose.

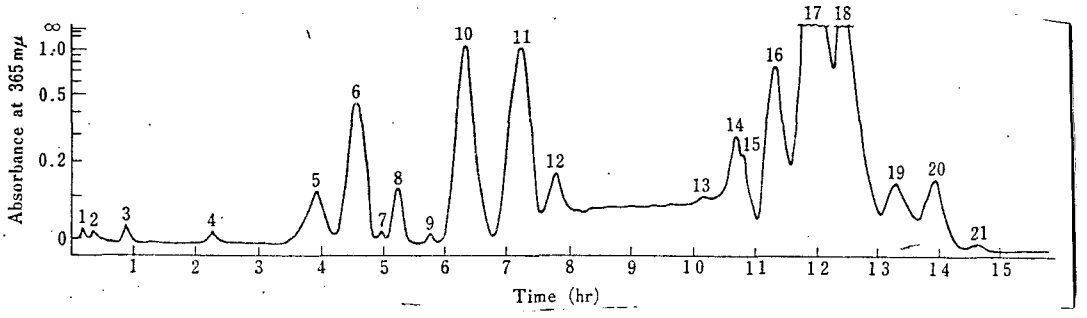

Figure 9. Liquid-Solid Chromatogram of Formose Sugar. Apparatus: Hitachi 034-0004-KLA-3B type Liquid Chromatograph. Column: 4.5x x50 cm, column temp. 55°C. Packing: Bio Rad AG 1x2. Sample: Formose 19.65 mg/0.5 ml. Eluent: 1. 0.25 M H_3BO_3, pH 8.2, 365 min. 2. 0.60 M H_3BO_3, pH 10.2. Flow rate effluent: 15 ml/hr (6.0 kg/ /cm^2); flow raterreagent: 45 ml/hr (5.9 kg/cm^2). Reagent: phosphoric acid + acetic acid + aniline. Reaction bath temp: 120°C, reaction coil: ϕ0.8x23 m. Cell path: 2 mm, wave length: 365 mμ. Chart speed: 6 cm/hr. Scale: 0-0.2 expansion.

From the above results, hexose constitutes the principal sugar among the formose sugar components. The secondary component sugar is pentose, while such microscopic component sugars as triose, tetrose and heptose, etc. are present. It was possible to classify such formose components sugars (all DL-racemic bodies)

as glycerose, dioxyacetone, erytherose, threose, arabinose, xylose, mannose, galactose, glucose, gulose, sorbose and fructose, etc. In addition, other unclassified sugarsspots and peaks were detected. These will be the subject of future study.

SUMMARY

A study was made of formose component sugars by applying various types of /350 chromatography (PPC, TLC, GLC, LSC, etc.) to formose and its derivatives. The following results were obtained. Formose is a mixture of monosaccharides containing hexose as the principle sugar and pentose as the secondary sugar and minor quantities of triose, tetrose, heptose, etc. Among these sugars, glycerose and dioxyacetone were identified as the C_3 sugars, erythrose and threose as the C_4 sugars, arabinose and xylose as the C_5 sugars, mannose, galactose, glucose, gulose, sorbose, and fructose, etc. as the C_6 sugars. All of these were the DL-racemic bodies.

In conclusion, thanks are expressed to Dr. N. K. Richtmyer of the American National Institute of Health and Dr. Hirao Okuda, Professor, College of Medicine, Okayama University for donating the important seven carbon sugar samples. In addition, deep thanks are extended to Dr. Professor E. Pfeil of the German Chemical Institute of the University of Marburg and Dr. Professor K. Runge of the Organic Chemistry Institute of the Dresden Technical University for their instruction on the formose formation reaction.

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